A Hierarchical NGF Signaling Cascade Controls Ret-Dependent and Ret-Independent Events during Development of Nonpeptidergic DRG Neurons

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SUMMARY

NGF controls survival, differentiation, and target innervation of both peptidergic and nonpeptidergic DRG sensory neurons. The common receptor for GDNF family ligands, Ret, is highly expressed in nonpeptidergic neurons, but its function during development of these neurons is unclear. Here, we show that expression of Ret and its coreceptors GFRα1 and GFRα2 is dependent on NGF. GFR/Ret signaling, in turn, autoregulates expression of both GFRα1 and GFRα2 and promotes expression of TrpA1, MrgA1, MrgA3, and MrgB4, acquisition of normal neuronal size, axonal innervation of the epidermis, and postnatal extinction of the NGF receptor TrkA. Moreover, NGF controls expression of several other genes characteristic of nonpeptidergic neurons, such as TrpC3, TrpM8, MrgD, and the transcription factor Runx1, via a Ret-independent signaling pathway. These findings support a model in which NGF controls maturation of nonpeptidergic DRG neurons through a combination of GFR/Ret-dependent and -independent signaling pathways.

INTRODUCTION

Sensory neurons of the dorsal root ganglia (DRG) detect information in the periphery and convey it to the central nervous system. DRG neurons are greatly diversified with respect to both morphological and physiological properties. Subsets of these neurons respond to tactile, thermal, proprioceptive, and nociceptive stimuli and are classified as mechanoreceptors, thermoreceptors, proprioceptors, and nociceptors, respectively. In addition to detecting distinct sensory modalities, each population expresses a unique array of molecular markers, including ion channels and receptors for specific neurotrophic growth factors. A central question in sensory neurobiology is how such remarkable diversity of primary somatosensory neurons is achieved during development.

There are two main populations of cutaneous nociceptors in adult mice (Molliver et al., 1995; Priestley et al., 2002). One population expresses the tyrosine kinase Ret, the signaling receptor for the glial-derived neurotrophic factor (GDNF) family ligands (GFLs), and comprises approximately 60% of total DRG neurons. The other, smaller population expresses TrkA, the receptor for nerve growth factor (NGF). Mature TrkA+ neurons express the neuropeptide calcitonin gene-related peptide (CGRP) and are accordingly termed peptidergic nociceptors. Ret+ neurons, in contrast, are called nonpeptidergic nociceptors and are characterized by their ability to bind to the lectin Griffonia simplicifolia IB4. These two populations exhibit distinct patterns of peripheral and central axonal projections. The peripheral axons of TrkA+ peptidergic neurons terminate in the stratum spinosum (SS) of glabrous skin while their central axonal projections terminate in lamina I and outer lamina II (IIO) of the dorsal horn of the spinal cord. Peripheral projections of Ret+ nonpeptidergic neurons, in contrast, terminate in the superficial stratum granulosum (SG) of the epidermis, while their central axonal projections end in inner lamina II (IIi) of the spinal cord (see Figure S2A in the Supplemental Data available with this article online; Zylka et al., 2005).

DRG neurons arise from neural crest precursors which, in the mouse, coalesce into ganglia beginning around E9.5. Shortly after neurogenesis, most DRG neurons initiate expression of TrkA and become responsive to NGF (White et al., 1996). A distinct but small population of TrkA− DRG neurons expresses Ret as early as E12; these TrkA−/Ret+ neurons are classified as the early Ret population (Molliver et al., 1997). The vast majority of Ret+ neurons, however, emerge from TrkA+ neurons during the late embryonic period. These neurons begin to express Ret around E16 and gradually extinguish expression of TrkA.
after birth (Molliver and Snider, 1997; Molliver et al., 1997). Thus, although TrkA is expressed in most DRG neurons during embryonic development, only a subset of them, mainly CGRP+ peptidergic neurons, continue to express TrkA into adulthood. The segregation of TrkA+ and Ret+ neuronal populations is complete between 2 and 3 weeks after birth.

What are the factors that control the differentiation and segregation of TrkA+ and Ret+ nociceptors? Neurotrophic factors, including NGF, BDNF, and NT-3, along with their cognate receptors, are critical for survival, differentiation, and peripheral target innervation of distinct classes of DRG sensory neurons (Marmigere and Ernfors, 2007). A well-documented example is the dependence of nociceptor development on NGF-TrkA signaling (Crowley et al., 1994; Levi-Montalcini, 1987). The transition of nonpeptidergic neurons from TrkA+ to Ret+ suggests that these neurons switch their dependence from TrkA signaling to Ret signaling during postnatal periods (Molliver et al., 1997). However, whether Ret signaling controls differentiation, survival or other aspects of development of nonpeptidergic neurons remains unclear mainly because Ret null mice die shortly after birth due to renal agenesis, prior to final maturation of these neurons.

The GDNF family of ligands (GFLs) contains four members, GDNF (Lin et al., 1993), neurturin (NRTN; Kotzbauer et al., 1996), artemin (ARTN; Baloh et al., 1998), and persephin (PSPN; Milbrandt et al., 1998). Each GFL signals through a receptor complex containing the signaling subunit Ret and one member of a family of GPCR-like proteins, the GFR receptors (GFRs). There are four GFRs in vertebrates, designated GFRα1 through GFRα4. In vitro binding assays indicate that GDNF binds preferentially to GFRα1 (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996), NRTN to GFRα2 (Buj-Bello et al., 1997; Klein et al., 1997), ARTN to GFRα3 (Baloh et al., 1998), and PSPN to GFRα4 (Enokido et al., 1998). GFRα1, GFRα2, GFRα3, but not GFRα4 are expressed in DRG neurons. There are inconsistencies among the reported DRG phenotypes of the various GFL and GFR null mice (Cacalano et al., 1998; Enomoto et al., 1998; Honma et al., 2002; Lindfors et al., 2006; Moore et al., 1996; Nishino et al., 1999; Rossi et al., 1999), which are potentially explained by either redundant roles of GFRs and/or GFLs or unpredicted GFL-GFR interactions in vivo. Thus, conditional ablation of Ret in sensory neurons is required to establish the functions of GFL-GFR/Ret signaling in development of nonpeptidergic sensory neurons in vivo.

In addition to neurotrophic growth factors, several transcription factors are implicated in the control of nociceptor differentiation (Marmigere and Ernfors, 2007). Neurogenin 1 (Ngn1) and Neurogenin 2 (Ngn2) are required soon after DRG formation for the generation of sensory neurons, including nociceptors (Ma et al., 1998, 1999). The Runt domain transcription factor Runx1 is also critical for nociceptor differentiation (Chen et al., 2006; Kramer et al., 2006b; Yoshikawa et al., 2007). Runx1 is essential for expression of Ret, certain Mrg and Trp genes, and other genes characteristic of Ret+ nonpeptidergic nociceptors. However, the identity of upstream factors that regulate Runx1 and other genes that define the molecular, morphological, and functional properties of Ret+ nociceptors are unknown.

Here, we report that NGF regulates expression of GFRs and Ret, instructing a subset of TrkA+ sensory neurons to adopt the nonpeptidergic sensory neuron fate. We show that Ret signaling is critical for acquisition of several features of the nonpeptidergic neuronal phenotype, including expression of a subset of Trp and Mrg genes, innervation of the epidermis, control of normal neuronal size, and postnatal extinction of TrkA. Interestingly, expression of some other Trp and Mrg genes, also characteristic of Ret+ nociceptors, is independent of Ret signaling. Instead, NGF controls their expression, possibly through regulation of Runx1. Our findings support a model in which NGF promotes differentiation and maturation of nonpeptidergic neurons through both Ret-dependent and Ret-independent signaling mechanisms.

**RESULTS**

**Developmental Transition of Nonpeptidergic DRG Neurons from TrkA+ to Ret+**

To examine the process of differentiation of DRG nociceptor neurons and, in particular, the emergence of Ret+ nonpeptidergic nociceptors from TrkA+ precursors, we first performed double fluorescent in situ hybridization on wild-type mouse DRGs at embryonic day 12 (E12), E16, postnatal day 0 (P0), P5, P10, and P14 using cRNA probes against TrkA and Ret (Figure 1A). TrkA expression is readily detected in the majority of DRG neurons at E12. In contrast, expression of Ret is observed in only a few neurons at E12, attributable to the previously described early Ret+ neuronal population (Molliver et al., 1997). These early Ret+ neurons have large-diameter soma and do not coexpress TrkA (Figure 1A). Many TrkA+ neurons, however, begin to coexpress Ret at E16 (Figure 1A). These small to medium diameter Ret+ neurons are the late Ret+ population destined to become nonpeptidergic nociceptors, which constitute more than 90% of the total Ret+ DRG neurons of the adult mouse. These Ret+/TrkA+ neurons undergo a phase of TrkA extinction, which begins postnatally and is not complete until P14 (Figure 1A). Our in situ hybridization findings are consistent with previous observations (Molliver and Snider, 1997; Molliver et al., 1997) and suggest that nonpeptidergic nociceptors undergo a transition from dependence on NGF-TrkA signaling to GFL-GFR/Ret signaling.

**GFRα2 Is the Predominant Coreceptor Expressed in Ret+ DRG Neurons**

Despite previous reports describing the expression of GFRα1, GFRα2, and GFRα3 in DRG neurons, the relative importance of GFR coreceptors for Ret signaling in DRGs is unknown (Fundin et al., 1999). We performed double fluorescent in situ hybridization analysis of DRGs at P14 using cRNA probes directed against each of the GFRs
and Ret. $GFR\alpha1$ is expressed in 18.2% ± 5.5%, $GFR\alpha2$ in 61.4% ± 6.9%, and $GFR\alpha3$ in 13.9% ± 4.0% of Ret+ neurons (Figures 1B–1D). We also performed similar analysis with probes against all GFRs and Ret and found the degree of overlap to be 99% ± 2% (Figure 1E). Together, these results suggest that the overlap of expression of different GFRs, if any, is minimum at P14.

Interestingly, while virtually all $GFR\alpha2^+$ neurons strongly coexpress Ret, many of the $GFR\alpha1^+$ and $GFR\alpha3^+$ neurons weakly coexpress Ret. Also, expression of $GFR\alpha2$ in DRG...
neurons increases dramatically after birth (Figure S1), which is temporally coincident with extinction of TrkA in nonpeptidergic neurons. In addition, unlike GFRα2−/− mice, neither GFRα1−/− nor GFRα3−/− mice show any deficits in DRG neurons (Cacalano et al., 1998; Lindfors et al., 2006; Nishino et al., 1999). These findings indicate that GFRα2 is the predominant coreceptor of Ret in postnatal DRG sensory neurons.

NGF Controls Expression of Ret and Its Coreceptors

NGF−/−;Bax−/− mice have proven to be valuable tools to study survival-independent functions of NGF (Patel et al., 2000). While nearly all embryonic TrkA+ neurons in NGF−/− mice die by apoptosis, these neurons in NGF−/−;Bax−/− mice survive in the absence of NGF due to the concomitant loss of the proapoptotic gene Bax. TrkA+ neurons are, however, hypertrophic in this double-mutant mouse line. The TrkA+ neurons in NGF−/−;Bax−/− mice, on the other hand, appear normal. Since the majority of Ret+ nociceptors emerge from immature TrkA+ neurons, we sought to determine whether NGF controls expression of Ret and its GFR coreceptors in these neurons. To address this possibility, we compared the expression of Ret, GFRα1, GFRα2, and GFRα3 in P0 DRGs from NGF−/−;Bax−/− mice and NGF−/−;Bax−/− control mice. Since NGF−/−;Bax−/− mice die shortly after birth, our analysis was restricted to this time point. In agreement with a previous report (Patel et al., 2000), Ret expression is dramatically reduced in NGF−/−;Bax−/− DRGs; there are only a few strongly Ret+ neurons of normal soma size in double-mutant mice (Figures 2A and 2B). These few remaining Ret+ neurons belong to the early Ret+ population because they do not coexpress TrkA (Figures S2B and S2C). In addition, expression of GFRα1 is undetectable in DRGs from NGF−/−;Bax−/− mice (Figures 2C and 2D). Moreover, although expression of GFRα2 is normally weak at P0 and increases postnatally in control mice, a dramatic reduction in expression of this receptor in DRGs of NGF−/−;Bax−/− mice was observed (Figures 2E and 2F). GFRα3, on the other hand, does not appear to be regulated by NGF, despite its expression in hypertrophic neurons of NGF−/−;Bax−/− mice (Figures 2G and 2H). Cell culture and RT-PCR were also done to ask whether NGF directly stimulates expression of Ret and its GFR coreceptors in sensory neurons. In agreement with the in vivo results, NGF was found to induce expression of Ret, GFRα1, GFRα2, but not GFRα3 in cultured sensory neurons (Figures 2I and 2J). These observations, taken together, indicate that NGF controls expression of both Ret and its coreceptors, GFRα1 and GFRα2, in nonpeptidergic nociceptor neurons.

Ret Conditional Knockout Mice

The roles of Ret signaling in sensory neurons have been difficult to discern due, at least in part, to the perinatal lethality of Ret null mice (Schuchardt et al., 1994). To overcome this obstacle, and to examine the influence of Ret signaling pathways on postnatal development of nonpeptidergic sensory neurons, mice carrying a LoxP-based conditional Ret allele were generated (see Experimental Procedures and Figure S3A). Mice harboring the conditional Ret allele (Retfl/+;Wnt1-Cre mice) were crossed with mice carrying a Wnt1-Cre transgene (Chai et al., 2000), which directs expression of Cre recombinase in premigratory neural crest cells, including all progenitors of DRG neurons. Mice heterozygous for both the floxed Ret allele and the Wnt1-Cre allele (Retfl/+;Wnt1-Cre mice) are viable and fertile and exhibit no obvious deficits. Importantly, Ret expression is undetectable in DRG neurons in Retfl/+;Wnt1-Cre mice (Figures 3A and 3B), indicating efficient Cre-mediated excision of the floxed Ret allele. The exquisite specificity of Cre-mediated recombinase is shown by the unperturbed expression of Ret in motor neurons in the ventral horn of the spinal cord (Figures 3A and 3B). In keeping with Cre expression in the neural crest, we also observed complete loss of Ret expression in the myenteric plexi and sympathetic ganglia of Retfl/+;Wnt1-Cre mice (Figures S3C and S3D and data not shown). Grossly, Retfl/+;Wnt1-Cre mice are indistinguishable from their littermates at birth. However, within 3 days of birth, these mice show abdominal distension, progressive weakness, and few survive beyond 3 weeks of age (Figure S3B). Examination of Retfl/+;Wnt1-Cre mice as late as P14 reveals that they have an enlarged jejunum, ileum, and colon, suggesting that they may have intestinal aganglionosis similar to human patients carrying Ret mutations who develop Hirschsprung’s disease (data not shown). To further characterize this phenotype, the small intestine and colonic wall were isolated from mutant mice and whole-mount acetylcholinesterase histochemistry was performed to visualize enteric neurons. In agreement with previous findings that Ret is required for migration and survival of enteric neurons (Natarajan et al., 2002), a complete loss of enteric neurons in both the small intestine and colon of Retfl/+;Wnt1-Cre mice was observed (Figures S3E and S3F). Retfl/+;Wnt1-Cre mice are therefore a useful animal model to address the embryonic and postnatal functions of Ret signaling in sensory, sympathetic, and enteric neurons in vivo.

Ret Is Not Required for Cell Viability but Is Required for Control of Neuronal Soma Size of Nonpeptidergic DRG Neurons

Initial analysis of DRGs from Retfl/+;Wnt1-Cre mice revealed that they are similar in size to those of Retfl/+ control littermates at P0 but are approximately 30% smaller at P14 (Figures S4A–S4D). We considered whether either neuronal loss or hypertrophy is responsible for this phenotype because these phenomena have been reported in GDNF−/− and GFRα2−/− mice, respectively (Lindfors et al., 2006; Moore et al., 1996).

To ask if cell viability is compromised in the absence of Ret signaling, we performed Nissl staining and cell counting on whole L5 DRGs from P14 Retfl/+ and Retfl/+;Wnt1-Cre mice. On average, mutant L5 DRGs yielded 30% fewer sections compared to controls (55 ± 4 sections in mutants versus 80 ± 4 sections in controls, n = 3). Our initial
analysis revealed a 17.6% deficit in the number of neurons in Ret<sup>-/-</sup>;Wnt1-Cre mice compared to Ret<sup>-/-</sup> animals (Figure 3K). However, when cell counts of dissociated DRG neurons were performed, no significant difference in neuronal numbers between DRGs from Ret<sup>-/-</sup> and Ret<sup>-/-</sup>;Wnt1-Cre mice was found (95.6% ± 2.5% of control). In addition, active caspase-3-positive neurons were not observed at P10 in either mutant or control DRGs in vivo, suggesting neuronal viability is not affected in the absence of Ret postnatally (Figures S4E and S4F). Furthermore, the distribution of several marker genes expressed in peptidergic nociceptors (CGRP), nonpeptidergic nociceptors (P2X3) and mechanoreceptors (NF200) was analyzed. We reasoned that loss of nonpeptidergic neurons should be reflected by a higher proportion of CGRP<sup>+</sup> neurons and a lower proportion of P2X3<sup>+</sup> neurons in DRGs of Ret<sup>-/-</sup>;Wnt1-Cre mice. In fact, the proportions of these markers are nearly identical for DRGs from control and Ret<sup>-/-</sup>;Wnt1-Cre mice at P14 (Figure 3O). These findings together argue against the possibility of loss of nonpeptidergic neurons in Ret<sup>-/-</sup>;Wnt1-Cre DRGs.

To investigate the possibility of neuronal hypotrophy in Ret<sup>-/-</sup>;Wnt1-Cre DRGs, immunohistochemistry and cell size analysis of peripherin<sup>+</sup>, CGRP<sup>+</sup>, and neurofilament 200<sup>+</sup> DRG neurons was performed. Peripherin is a neurofilament-associated protein expressed selectively in most small- and medium-diameter DRG neurons, including both peptidergic and nonpeptidergic cutaneous neurons.
Figure 3. Ret Signaling Is Dispensable for Survival but Is Required for Hypertrophy of Nonpeptidergic Neurons

(A and B) Loss of Ret expression in sensory neurons but not motor neurons (white arrowheads) of P0 Ret$^{ff}$;Wnt1-Cre mice (n = 3). Scale bar, 100 μm.

(C and D) Peripherin immunostaining in DRGs of Ret$^{ff}$ and Ret$^{ff}$;Wnt1-Cre animals at P14. Note the hypotrophic neuronal soma of most peripherin$^+$ neurons in the mutant animal.

(E and F) Neurofilament-200 immunostaining of DRG neurons demonstrates that large diameter neurons have normal cell size in the absence of Ret. (G and H) CGRP immunostaining in the Ret$^{ff}$ and Ret$^{ff}$;Wnt1-Cre mouse DRGs shows comparable cell sizes in control and mutant animals.

(I and J) Double-immunofluorescent staining of P2X3 (red) and peripherin (green) (n = 3). Scale bar, 20 μm. Similar numbers of double-positive neurons are observed in control and mutant animal DRGs. However, double-labeled neurons have a significantly smaller soma size in the mutant DRGs compared to control animals (see arrowheads, inset). Inset scale bar, 20 μm.

(K) Cell counts observed by Nissl staining and whole-cell counting. Total neuronal profiles scored were from L5 DRGs of pooled P10 (n = 1) and P14 animals (n = 3) for each condition. For Ret$^{ff}$ animals, a total of 17,312 ±1,423 neurons per L5 DRG and for Ret$^{ff}$;Wnt1-Cre animals a total of 14,265 ± 941 neurons per L5 DRG were scored.

(L–N) Cell size histograms displaying the distribution of mean soma area for neurons labeled with peripherin (L), neurofilament-200 (M), and CGRP (N).

(O) The relative ratios of neurofilament 200, peripherin, CGRP, and P2X3 positive neurons per DRG normalized against PGP9.5$^+$ DRG neurons in Ret$^{ff}$ and Ret$^{ff}$;Wnt1-Cre mice.

Shown are means ± SEM.
Most peripherin\(^+\) DRG neurons in P14 Ret\(^{ff}\);Wnt1-Cre mice are significantly smaller (47.3\% reduction in soma area) compared to those in control Ret\(^{ff}\) mice (Figures 3C, 3D, and 3L). This reflects hypotrophy only in nonpeptidergic neurons because the soma sizes of CGRP\(^+\) peptidergic neurons are similar in Ret\(^{ff}\);Wnt1-Cre and control mice (Figures 3G, 3H, and 3N). Moreover, neurofilament 200 (NF200)-positive neurons, which are large-diameter proprioceptive neurons, have comparable soma sizes in mutants and controls (Figures 3E, 3F, and 3M). Finally, double-fluorescent immunostaining for peripherin and P2X3 was performed. Neurons labeled with both markers are mostly nonpeptidergic neurons, and these neurons clearly have smaller soma sizes in Ret\(^{ff}\);Wnt1-Cre mice compared to controls (Figures 3I and 3J, see inset). Thus, the absence of Ret signaling leads to hypotrophy of nonpeptidergic nociceptors, whereas peptidergic nociceptors and proprioceptive neurons are unaffected. Taken together, these findings indicate that Ret signaling is required for the acquisition of normal soma size but not survival of nonpeptidergic DRG nociceptors.

**Loss of Ret Signaling Influences Peripheral but Not Central Projections of Nonpeptidergic Sensory Neurons**

We next examined the nature of peripheral and central axonal projections in Ret\(^{ff}\);Wnt1-Cre mice. For these analyses, we used an MrgD-EGFP reporter mouse line, which has been previously shown to express GFP in most, if not all, nonpeptidergic nociceptive fibers innervating the epidermis (Zylka et al., 2005). We crossed the Ret\(^{ff}\) mice to this MrgD-EGFP mouse line to obtain Ret\(^{ff}\);MrgD-EGFP control and Ret\(^{ff}\);Wnt1-Cre;MrgD-EGFP mutant mice. As expected, GFP\(^+\) fibers are abundant in the epidermis of Ret\(^{ff}\);MrgD-EGFP control mice, with free nerve endings terminating in the stratum granulosum. CGRP\(^+\) fibers, on the other hand, were found mostly restricted to the subepidermal layer, with a few free nerve endings coursing through the epidermis and terminating in the stratum spinosum (Figures 4A, 4C, 4E, and 4G). In the Ret\(^{ff}\);Wnt1-Cre;MrgD-EGFP mutant mice, although the CGRP\(^+\) innervation to the epidermis was present, there was a substantial reduction in the number of GFP\(^+\) nonpeptidergic fibers innervating the epidermis (Figure 4K). Furthermore, the few remaining GFP\(^+\) fibers in the epidermis of mutant mice were weakly stained in a punctate manner (Figures 4B, 4D, 4F, and 4H). In complementary experiments, we employed PGP9.5 and CGRP to label nonpeptidergic sensory fibers by exclusion (Lindfors et al., 2006) and found a similar phenotype (Figure S5). The lack of nonpeptidergic epidermal fibers in Ret\(^{ff}\);Wnt1-Cre mice, found by these two methods, could be caused by a failure of epidermal innervation, axonal retraction, severe axonal hypotrophy, or a combination of the above.

To examine whether central projections of nonpeptidergic neurons are affected by the absence of Ret function, we performed CGRP and GFP double immunostaining on spinal cord sections from Ret\(^{ff}\);MrgD-EGFP and Ret\(^{ff}\);Wnt1-Cre;MrgD-EGFP mice. Central projections of both CGRP\(^+\) and GFP\(^+\) neurons formed distinct layers, and the lamina-specific innervation of nonpeptidergic neurons is unperturbed in Ret\(^{ff}\);Wnt1-Cre;MrgD-EGFP mice (Figures 4I, 4J, and 4L). Thus, Ret signaling is essential for cutaneous peripheral projections of nonpeptidergic neurons, but it is not required for the integrity of their central projections.

**Ret Signaling Controls Expression of Its Coreceptors**

Although a 45\% reduction of GFR\(_2\)^+ neurons was reported in NRTN\(^{-/-}\) DRGs (Heuckeroth et al., 1999), no cell loss was found in GFR\(_2\)^{ff} mice (Lindfors et al., 2006). Since we do not observe loss of nonpeptidergic neurons in Ret mutant DRGs, we speculate that the reduction of GFR\(_2\)^+ neurons reported for NRTN\(^{-/-}\) DRGs may not be due to cell death. Instead, the deficit could be explained by a reduction in the level of GFR\(_2\)^{ff}. To examine whether Ret signaling indeed regulates expression of GFR\(_2\)^{ff} or other GFRs, we assessed the expression of GFRs in Ret\(^{ff}\);Wnt1-Cre DRGs at both P2 and P10 by in situ hybridization (Figures 5 and S6). The number of cells expressing GFR\(_1\) and GFR\(_2\) is significantly reduced in Ret\(^{ff}\);Wnt1-Cre mice (60\% and 25\% reduction for GFR\(_1\) and GFR\(_2\), respectively), suggesting that expression of these coreceptors is controlled by Ret signaling itself. Because there is a near-complete elimination of GFR\(_1\) and GFR\(_2\) expression in DRG neurons of NGF\(^{-/-}\);Bax\(^{-/-}\) DRGs at P0 (Figures 2C–2F), these findings suggest that NGF controls initial expression of GFR\(_1\) and GFR\(_2\) and Ret signaling then autoregulates expression of these GFR coreceptors.

**Ret Signaling Controls Differentiation of Nonpeptidergic DRG Neurons**

One interesting possibility is that NGF-dependent expression of Ret and its GFR coreceptors enables a subset of immature TrkA\(^+\) neurons to undergo GFL-dependent differentiation and maturation. To determine whether Ret signaling controls final stages of differentiation of nonpeptidergic nociceptors, we evaluated the expression of a panel of genes that define the physiological properties of nonpeptidergic neurons in DRGs from control and Ret\(^{ff}\);Wnt1-Cre mice.

Trp class ion channels have been implicated as versatile cell sensors in multiple sensory modalities, including nociception and thermal sensation (Caterina, 2007). Expression of TrpA1 is completely absent in Ret\(^{ff}\);Wnt1-Cre DRGs at P14, while expression of TrpC3, TrpM8, and TrpV1 is unaffected (Figures 6A–6H). The Mrg family includes more than fifty G protein-coupled receptors (GPCRs), a dozen of which are exclusively expressed in subsets of Ret\(^+\) nonpeptidergic sensory neurons (Dong et al., 2001). In P14 Ret\(^{ff}\);Wnt1-Cre DRGs, expression of MrgA1, MrgA3, and MrgB4 is greatly reduced or absent, while expression of MrgD is unperturbed compared to controls (Figures 6K–6R). The expression of MrgB4 is normally initiated at P4, and, in Ret mutant mice, expression of this gene was

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almost completely lost by P6 (Figure S7). This observation is consistent with the idea that Ret signaling is important for initiation of expression of MrgB4 rather than for its maintenance. In contrast, expression of P2X3 in mutant DRGs is similar to that of controls (Figures 6I and 6J). These results indicate that Ret signaling controls expression of a subset of genes characteristic of mature nonpeptidergic sensory neurons.

Expression of TrpA1, TrpC3, TrpM8, TrpV1, MrgB4, MrgD, and P2X3 is either reduced or eliminated in Runx1−/− DRGs (Chen et al., 2006) and, as shown above, expression of a subset of these genes is also lost in the absence of Ret signaling. We therefore asked whether the phenotypes observed in Retf/f;Wnt1-Cre mice are caused by loss of expression of Runx1. To test this idea, expression of Runx1 in Retf/f;Wnt1-Cre and control DRGs was assessed and found to be unaffected (Figures 6S and 6T). Consistent with this observation, Runx1 expression begins at around E12.5, whereas Ret expression in nonpeptidergic neurons is initiated at E16. Therefore, Ret
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Retf/f;Wnt1-Cre

ing neurons is lower in to a reduction in the number of cells expressing GFR36 ± 3 positive neurons/section) in DRGs at P10. Note that in addition of each genotype. Scale bar, 40

functions downstream of Runx1, and loss of Ret expression in Runx1−/− mice (Chen et al., 2006) likely accounts for reduced expression of TrpA1 and MrgB4 in these mutants.

Expression of Ret, Its Coreceptors, and Other Marker Genes Is Regulated by NGF, Possibly through a Runx1-Dependent Pathway

A unique repertoire of genes is expressed in Ret+ nociceptors, and our findings indicate that expression of a subset of them is controlled by Ret signaling. It is noteworthy that expression of many Ret-independent genes including TrpM8 is initiated prior to expression of Ret (Chen et al., 2006). These observations led us to ask whether extracellular signals other than GFLs control expression of these Ret-independent genes. Since many genes expressed in nonpeptidergic nociceptors are regulated by Runx1, a related question is as follows: do extracellular cues promote expression of Runx1 enabling a subset of TrkA+ neurons to adopt a nonpeptidergic neuronal fate? NGF stands out as a candidate because its receptor TrkA is expressed as early as E11.5, and NGF controls expression of Ret, which is also controlled by Runx1 (Chen et al., 2006). To address whether NGF controls expression of genes characteristic of nonpeptidergic neurons and Runx1, we examined expression of TrpC3, TrpM8, TrpV1, MrgA1, MrgA3, MrgD, P2X3, and Runx1 in both NGF+/−;Bax+/− and NGF+/−;Bax−/− control DRGs at P0 by in situ hybridization (Figure 7). Remarkably, expression of TrpC3, MrgA1, MrgA3, and MrgD is completely eliminated (Figures 7A–7F, 7I, and 7J) and TrpM8 is greatly reduced (Figures 7K and 7L) in NGF+/−;Bax+/− DRGs compared to NGF+/−;Bax−/− controls. In contrast, expression of TrpV1 is only slightly reduced (Figures 7M and 7N), and expression of P2X3 is unaffected (Figures 7G and 7H) in NGF+/−;Bax−/− mutants. Importantly, expression of Runx1 itself is dramatically compromised in NGF+/−;Bax−/− DRGs (Figures 7O and 7P) at P0. These results show that Runx1 expression is under the control of NGF. Interestingly, expression of Runx1 is unaffected in DRGs from NGF−/−;Bax−/− mice at E14 (Figures 7Q and 7R), suggesting that NGF is not required for initiation of Runx1 expression but is critical for its maintenance. Since expression of most genes examined is not initiated until late in development, it is therefore possible that NGF controls expression of these genes through a Runx1-dependent transcriptional pathway.

Ret Is Required for the Postnatal Extinction of TrkA

A critical, late event in maturation of Ret+ nonpeptidergic nociceptors is the postnatal extinction of TrkA (Figure 1; Molliver and Snider, 1997). Since expression of Ret precedes extinction of TrkA, we hypothesized that NGF-dependent expression of Ret and its coreceptors enables GFL-GFR/Ret signaling to instruct sensory neurons to extinguish TrkA. To address this possibility, we examined the postnatal time course of TrkA extinction in control and Retf/f;Wnt1-Cre mice. In our analysis of TrkA expression from P0 to P14, we observed TrkA extinction in both control and mutant animals, but to different extents. At P0, prior to the onset TrkA extinction, levels of TrkA expression in DRGs from both control and Retf/f;Wnt1-Cre mice are indistinguishable (Figures 8A, 8B, and 8K). By P14, however, the number of TrkA+ neurons had dramatically decreased in control DRGs while, in contrast, the percentage of TrkA+ neurons remained significantly higher in mutant DRGs. This difference is evident both at the mRNA and protein levels (Figures 8C–8F and 8K and data not shown). We reasoned that if the increase in number of TrkA+ neurons in Retf/f;Wnt1-Cre DRGs is due to a failure of immature nonpeptidergic neurons to extinguish TrkA, then this deficit should be reflected by a higher proportion of cellular overlap between TrkA and the nonpeptidergic neuronal marker IB4 and a lower proportion of overlap between TrkA and the peptidergic neuronal marker CGRP at P14. Indeed, although only a few IB4+ neurons express TrkA in control P14 DRGs (9% ± 1.8%), the majority of IB4+ neurons (67% ± 9.1%) continue to express TrkA in Retf/f;Wnt1-Cre mice (Figures 8G, 8H, and 8L). Conversely, coimmunostaining with CGRP and TrkA revealed that nearly all TrkA+ neurons

Figure 5. Autoregulation of GFRs by Ret Signaling

(A–F) Expression of GFRα1 (Retf/f, 9 ± 2 positive neurons/section), GFRα2 (Retf/f, 78 ± 7; Retf/f;Wnt1-Cre, 50 ± 7 positive neurons/section), and GFRα3 (Retf/f, 41 ± 4; Retf/f;Wnt1-Cre, 36 ± 3 positive neurons/section) in DRGs at P10. Note that in addition to a reduction in the number of cells expressing GFRα1 and GFRα2, the level of expression of GFRα1, GFRα2, but not GFRα3 in the remaining neurons is lower in Retf/f;Wnt1-Cre mice. Shown are averages ±SEM from counts obtained from six to eight sections of three animals of each genotype. Scale bar, 40 μm. Similar results were observed in DRGs at P2 (Figure S6).
coexpress CGRP in control DRGs, whereas numerous small-diameter TrkA+ cells do not coexpress CGRP in Ret\textsuperscript{ff};Wnt1-Cre DRGs (Figures 8I, 8J, and 8L). These findings demonstrate that the increase in the percentage of TrkA+ neurons found in postnatal Ret\textsuperscript{ff};Wnt1-Cre mice is a consequence of the failure of immature Ret\textsuperscript{ff}/TrkA+ nonpeptidergic neurons to extinguish TrkA. Since TrkA extinction still partially occurs in the absence of Ret signaling, other signals must also play a role in this process. Nevertheless, NGF-dependent expression of Ret signaling is an important step in the postnatal extinction of TrkA, a late event in the maturation of nonpeptidergic sensory neurons.

**DISCUSSION**

Here, we show that NGF-dependent expression of the Ret receptor tyrosine kinase is required for diversification, maturation, and peripheral innervation of nonpeptidergic DRG sensory neurons. NGF controls expression of Ret and its coreceptors GFR\textalpha\textsubscript{1} and GFR\textalpha\textsubscript{2}. Ret signaling, in turn, regulates expression of both GFR\textalpha\textsubscript{1} and GFR\textalpha\textsubscript{2} and promotes expression of ion channels and receptors, acquisition of normal neuronal size, innervation of the epidermis, and extinction of TrkA. Moreover, NGF controls expression of other genes characteristic of nonpeptidergic

![Image of Figure 6.](https://example.com/figure6.png)
neurons, including Runx1, through a Ret-independent signaling pathway. We propose a model in which NGF instructs maturation of nonpeptidergic DRG neurons through a combination of GFR/Ret-dependent and independent signaling pathways (Figure 9).

**Ret Signaling Controls Cell Size but Not Survival of Nonpeptidergic Neurons**

Whether GFL-GFR/Ret signaling is required for survival of DRG neurons has been a controversial issue. There is a plethora of data supporting the idea that GFLs can support postnatal survival of DRG neurons in vitro (Adler, 1998; Forgie et al., 1999; Matheson et al., 1997; Molliver et al., 1997). However, of all the GFL and GFR mutant mice examined, only the GDNF and NRTN mutants are reported to exhibit a significant loss of DRG neurons (Heuckeroth et al., 1999; Moore et al., 1996). Our findings show that Ret signaling is dispensable for viability of nonpeptidergic DRG neurons in vivo. This conclusion is based on the following observations: (1) the number of Runx1+ neurons, which are destined to become nonpeptidergic neurons, is identical in Retf/f; Wnt1-Cre and Retf/f DRGs; (2) the number of neurons expressing P2X3, a marker of nonpeptidergic neurons, is virtually identical in Retf/f;
Wnt1-Cre and control DRGs; (3) no active caspase-3-positive neurons are observed in postnatal Retf/f;Wnt1-Cre DRGs; (4) counts of dissociated DRG neurons from mutant and control L5 DRGs reveal similar neuronal numbers; (5) the ratios of peptidergic, nonpeptidergic, and mechanosensory DRG neurons are comparable in mutant and control DRGs. We are unable to make a conclusion about the role of Ret signaling in the viability of the early Ret+ population, however, because of the lack of reliable markers to identify these neurons. Nevertheless, our

![Figure 8. Ret Signaling Is Required for Postnatal Extinction of TrkA](image-url)

(A–F) Immunostaining for TrkA in DRGs from Retf/f and Retf/f;Wnt1-Cre mice at P0 (A and B), P10 (C and D), and P14 (E and F) (n = 3). Scale bar, 40 μm. (G and H) Double labeling with TrkA immunohistochemistry and IB4 binding at P14 in Retf/f and Retf/f;Wnt1-Cre mouse DRGs. Arrows indicate the TrkA+/IB4+ neurons in Retf/f;Wnt1-Cre DRGs, which are smaller and weakly labeling with IB4 compared to control animals.

(I and J) Double immunostaining with TrkA and CGRP at P14. Arrows indicate the TrkA+/CGRP+ neurons in Retf/f;Wnt1-Cre DRGs, which are probably hypotrophic nonpeptidergic neurons that failed to extinguish TrkA. Scale bar, 20 μm.

(K) Quantification of TrkA extinction in DRG neurons in Retf/f and Retf/f;Wnt1-Cre mice as a function of age. Shown is the percentage of TrkA+ neurons per DRG (TrkA+ neurons/PGP9.5+ neurons) in Retf/f and Retf/f;Wnt1-Cre mice at P0 (78.5% ± 8.9% versus 75.7% ± 4.0%), P10 (49.0% ± 4.9% versus 68.2% ± 4.4%), and P14 (30.5% ± 2.2% versus 55.4% ± 7.1%). Note that although there is some TrkA extinction in Retf/f;Wnt1-Cre DRGs from P0 to P14 (p < 0.001), it is significantly less compared to that of Retf/f mice at both P10 and P14 (**p < 0.001; n = 3 for each condition).

(L) Ratios of TrkA+/CGRP+ neurons/CGRP+ neurons (24.5% ± 2.2% versus 99.8% ± 2.5%) and TrkA+/IB4+ neurons/IB4+ neurons (9% ± 1.8% versus 67.3% ± 9.1%) in Retf/f and Retf/f;Wnt1-Cre DRGs at P14 (**p < 0.001).

Shown are means ± SEM.
findings show that Ret signaling is not required for viability of nonpeptidergic neurons but is critical for acquisition of normal neuronal size.

**Which GFRs and GFLs Interact with Ret in Nonpeptidergic Neurons?**

Which are the relevant GFL-GFR interaction(s) that acti-
vate(s) Ret signaling during development of nonpeptider-
getic neurons? We found that GFRs and GFLs Interact with Ret
in Nonpeptidergic Neurons? Which are the relevant GFL-GFR interaction(s) that acti-
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**Which GFRs and GFLs Interact with Ret in Nonpeptidergic Neurons?**

Which GFRs and GFLs Interact with Ret in Nonpeptidergic Neurons? We found that Ret signaling is not required for viability of nonpeptidergic neurons but is critical for acquisition of normal neuronal size.

**Ret Signaling Is Required for Expression of Several Ion Channels and GPCRs in Nonpeptidergic Neurons**

The transcription factor Runx1 plays an essential role in the differentiation of nonpeptidergic neurons (Chen et al., 2006). In Runx1−/− DRGs, immature TrkA+ sensory neu-
rons fail to adopt a nonpeptidergic neuronal phenotype and instead acquire characteristics of peptidergic cutane-
ous neurons. The authors of that study contended that regulation of many nociceptive ion channels is Runx1 de-
pendent but Ret signaling independent, based on the ob-
ervation that expression of a Runx1-dependent gene, TrpM8, is initiated prior to Ret in DRG neurons. Our results with Ret+/+;Wnt1-Cre mice show this to be true. Indeed, expres-
sion of TrpM8, TrpC3, TrpV1, MrgD, and P2X3 is un-
changed in Runx1 mutants. However, our work also shows that expression of several other nonpeptidergic neuron-
specific genes is strictly dependent upon Ret signaling. Expression of TrpA1, MrgA1, MrgA3, and MrgB4 is either completely eliminated or greatly reduced in Ret+/+;Wnt1-
Cre DRGs. Consistent with these findings, artemin over-
expression in mouse skin enhances expression of TrpA1 in DRG neurons (Elitt et al., 2006). Taken together, our results show that there are both Runx1-dependent/Ret-independent and Runx1-dependent/Ret-dependent pathways that control gene expression events in nonpept-
dergic neurons. We speculate that these two pathways work in a sequential manner: the Runx1-dependent/Ret-
independent pathway functions between E13 to P0 to control expression of TrpC3, TrpM8, TrpV1, MrgD, and P2X3 and innervation of the proper dorsal lamina of the spinal cord. The Runx1-dependent/Ret-dependent path-
way, in contrast, functions from E16 and beyond to regu-
late expression of TrpA1, MrgA1, MrgA3, and MrgB4, acquisition of normal neuronal size, skin innervation, and extinction of TrkA (Figure 9).

**NGF Regulates Expression of Runx1, GFRs, and Ret**

Runx1 is required for expression of nearly all genes char-
acteristic of nonpeptidergic neurons. Interestingly, while expression of Runx1 is unaffected in NGF−/−;Bax−/− mice at E14, it is substantially reduced at P0. Expression of Runx1 is virtually unaffected in DRGs lacking Ret. These findings, taken together, suggest that NGF controls mainte-
ance of Runx1 expression, which, in turn, is likely to support expression of Ret, the GFRs, and a large cohort of genes characteristic of nonpeptidergic neurons. In ad-
dition, NGF may control the expression or activity of other factors that are required for efficient Runx1-dependent transcription, perhaps including Runx1 transcriptional
coactivators or inhibitors. Thus, genes expressed in nonpeptidergic neurons fall into one of three categories based on the control of their expression: there is an NGF-dependent, Runx1-dependent, and Ret-independent subclass (TRPM8, TRPC3, MrgD, and TrpV1); an NGF-dependent, Runx1-dependent, and Ret-dependent subclass (TRPA1, MrgA1, MrgA3, and MrgB4); and a third subclass of NGF-independent, Runx1-dependent genes (P2X3 and TRPV1). However, data supporting the notion of this third class of genes are more equivocal. One confounding point is that expression of neither TRPV1 nor P2X3 is exclusive to nonpeptidergic neurons. Also, there is only a 50% loss of P2X3+ neurons, and expression of TRPV1 is reduced only in a subset of neurons in Runx1−/− mice (Chen et al., 2006). These observations imply that if Runx1 is partially lost, as we observed in DRGs from P0 NGF−/−; Bax−/− mice, then expression of TRPV1 and P2X3 should be modestly affected. Nevertheless, our findings indicate that target-derived NGF controls both Ret-dependent and Ret-independent steps in the maturation of nonpeptidergic neurons and that NGF signals are mediated, at least in part, through maintenance of Runx1 expression.

The development of nonpeptidergic neurons involves a two step process consisting of Ret expression in TrkA+ precursors, followed by postnatal extinction of TrkA in Ret+/TrkA+ neurons. It is interesting that NGF controls the initial expression of GFRs and Ret, indicating that the GFR/Ret holoreceptor complex is synthesized in DRG neurons upon innervation of target fields and the acquisition of target-derived NGF. We further demonstrate that Ret signaling controls the postnatal extinction of TrkA. This indicates that, through the functions of Ret, NGF suppresses the expression of its own receptor thereby rendering Ret+ neurons insensitive to further NGF influence.

What is the importance of hierarchical control of neurotrophic factor signaling during nonpeptidergic sensory neuron development? One idea stems from the observation that multiple populations of neurons use the same neurotrophic growth factor for axonal growth and target innervation. GDNF, for example, is required at branch points in the limbs, including the brachial plexus and in muscle tissue, to guide motor neurons to their final targets (Kramer et al., 2006a). Although axons of developing cutaneous sensory neurons and spinal motor neurons share common initial, proximal trajectories, they diverge at their distal trajectories to innervate skin and skeletal muscle, respectively. Thus, if cutaneous sensory neurons prematurely express GFR/Ret holoreceptors, they may be inappropriately influenced by proximal growth and guidance cues, such as GDNF in the brachial plexus. An NGF-inducible Ret and GFR expression paradigm ensures that sensory neurons remain unresponsive to GFLs en route to their specific target fields but gain sensitivity to target-derived GFLs only after divergence from motor neuron projections. Thus, through their actions at intermediate and final targets, a limited repertoire of neurotrophic factors can be employed by axons of disparate sets of developing neurons.

**EXPERIMENTAL PROCEDURES**

**Generation of RetCre/Wnt1-Cre and NGF−/−;Bax−/− Mice**

The Ret targeting vector was constructed using a BAC clone isolated from a 129J mouse genomic library (RPCI-22). The 7.4 kb Hind III-Age I fragment spanning exons 7 through 13 of Ret was used as the long arm and the 0.8 kb Hind III-Hind III in intron 15 as the short arm. The 1.4 kb Age I-Hind III fragment containing exons 14 and 15 served as the targeted sequence. In addition, a single nucleotide missense mutation was introduced to change valine 805 to alanine. This mutation is functionally silent but does sensitize the resulting protein to chemical inhibition of its kinase activity (data not shown). The targeted sequence was placed upstream of an FRT-Neo-FRT selection cassette, and the resulting sequence was flanked by loxP sites. The targeting construct was linearized and electroporated into 129.1 mouse embryonic stem cells. Resulting clones were selected with G418 (300 µg/ml), screened by PCR, and verified with Southern blotting using both internal and external probes. Four confirmed clones were injected into blastocyst. Three germline-transmitting chimeric animals were identified after crossing to C57BL/6 mice. These mice were mated to a strain expressing Cre recombinase under the control of the Wnt1 promoter (a generous gift from Dr. Henry Sucov, University of Southern California). NGF−/−;Bax−/− mice were generated as previously described (Glebova and Ginty, 2004). MrgD-EGFP mice were kindly provided by Dr. David Anderson (Caltech) and Dr. Xinzhong Dong (Johns Hopkins University).

**In Situ Hybridization and Immunohistochemistry**

Digoxigenin (DIG)-labeled cRNA probes were used for in situ hybridization. In situ hybridization probes directed against GFRα1, GFRα2, GFRα3, Runx1, and TrkA were amplified with gene specific sets of PCR primers and cDNA templates prepared from E14.5 mouse DRG. Probes for TRPA1, TRPV1, TRPC3, and TRPM8 were a kind gift from Dr. David Corey (Harvard Medical School). In situ probes for MrgA1, MrgA3, MrgD, MrgB4, P2X3, and Ret were kindly provided by Dr. Xinzhong Dong (Johns Hopkins University). Double-fluorescent in situ hybridization was performed using a combination of DIG-labeled and fluorescein-labeled cRNA probes as previously described (Dong et al., 2001).

For immunohistochemistry on frozen DRG sections, the antibodies used in this study were as follows: rabbit anti-TrkA (a gift from Dr Louis Reichardt, University of California, San Francisco, 1:1000), rabbit (Chemicon, 1:2000) or mouse anti-periherin (Chemicon, 1:500), rabbit anti-CGRP (Chemicon, 1:1000), rabbit anti-PGP9.5 (Chemicon, 1:1000), rabbit anti-P2X3 (Chemicon, 1:1000), and mouse anti-Neurofilament-200 (Sigma, 1:500). Secondary antibody incubations were performed with Alexafluor-546 or Alexafluor-488 conjugated secondary antibodies (Molecular Probes, 1:500). For immunostaining with hindpaw glabrous skin to visualize the epidermal innervation, we followed the protocol as described (Zylka et al., 2005).

**Supplemental Data**

The Supplemental Data for this article can be found online at [http://www.neuron.org/cgi/content/full/54/5/739/DC1/](http://www.neuron.org/cgi/content/full/54/5/739/DC1/).

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REFERENCES


Function of NGF and Ret in Nociceptor Development